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Molecular Cytogenetic Definition of the Chicken Genome: The First Complete Avian Karyotype

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ABSTRACT

Chicken genome mapping is important for a range of scientific disciplines. The ability to distinguish chromosomes of the chicken and other birds is thus a priority. Here we describe the molecular cytogenetic characterization of each chicken chromosome using chromosome painting and mapping of individual clones by FISH. Where possible, we have assigned the chromosomes to known linkage groups. We propose, on the basis of size, that the NOR chromosome is approximately the size of chromosome 22; however, we suggest that its original assignment of 16 should be retained. We also suggest a definitive chromosome classification system and propose that the probes developed here will find wide utility in the fields of developmental biology, DT40 studies, agriculture, vertebrate genome organization, and comparative mapping of avian species.

THE ability to karyotype an individual or species is fundamental for any genome-mapping effort as both genetic and physical maps are made with reference to chromosome position. A karyotype provides a wealth of information about the genetic makeup of an animal or cell line, *e.g.*, about disease status, infertility, or tumorigenesis, and is, in effect, a low-resolution map of the whole genome. For most species, chromosomes can be distinguished relatively easily by either classical (*e.g.*, G-banding) means or molecular cytogenetics. Birds (class Aves) are a notable exception to this because, typically, the diploid number is ~ 80 and because birds have many cytologically indistinguishable microchromosomes.

The majority of avian genomic studies focus on the chicken (*Gallus domesticus*) and the chicken genome-mapping project continues apace. The genetic map now contains ~ 2000 loci within 50 linkage groups, and it covers ~ 4000 cM (EMARA and KIM 2003). Over 235 of these loci have homology with known human or mammalian genes. The number of chicken protein sequences deposited in the SwissProt and the TrEMBL databases is between 1000 and 2000 and $>600,000$

chicken expressed sequence tags are deposited in the dbEST database. Large numbers of chicken full-length cDNAs are already being sequenced and it has been predicted that the chicken has 35,000 genes in total. A significant barrier to the progress of the chicken genome project, however, has been the fact that the chromosomes have not hitherto been fully classified and thus a large number of genes remain without a chromosomal assignment.

The chicken genome-mapping project is also developing a number of resources essential for the study of a range of scientific disciplines. DNA microarrays are being generated to study metabolic functions and immune responses (MIN *et al.* 2003; NEIMAN *et al.* 2003) and to analyze global gene expression in target tissues of chickens (COGBURN *et al.* 2003). There are also projects to target gene function by disrupting and gaining functions with the use of RNAi methods (HUDSON *et al.* 2002; PEKARIK *et al.* 2003). The increase in these genomic resources, easy access to the large chick embryo, and the application of sophisticated means such as RNA interference and morpholinos provide unique tools for testing gene function in all vertebrates. A resource that has been unavailable thus far, however, is a set of unique chromosome identifier probes.

Single nucleotide polymorphisms within chicken genes are being exploited for the generation of candi-

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date genes for quantitative traits (EMARA and KIM 2003). Chicken accounts for 20% of meat consumption and most egg consumption worldwide. There is consequently extensive research into >200 chicken quantitative trait loci encoding for disease susceptibility, immunology, leanness, egg production, etc. (LIU *et al.* 2001; MARIANI *et al.* 2001; TATSUDA and FUJINAKA 2001). Many highly inbred and recombinant inbred chicken lines have large, well-defined pedigrees; thus, chicken is a primary model for the study of quantitative inheritance in humans and other vertebrates (JEURISSEN *et al.* 2000; LEDUR *et al.* 2000; LE BIHAN-DUVAL *et al.* 2001). Mapping of quantitative traits, however, requires a chromosomal assignment and this has not yet been possible for traits that map to the smaller microchromosomes.

Chicken DT40 cell lines are avian-leukosis-virus-induced B cell lines that exhibit a high ratio of targeted to random integration of transfected DNA constructs at homologous loci (DHAR *et al.* 2001). They are suitable as a model for recombination analysis in vertebrates and are being successfully used in gene disruption experiments (WINDING and BERCHTOLD 2001). A feature of DT40 cell lines, however, is that they have a high degree of chromosomal rearrangements that, to date, could not be karyotyped.

Finally, there is widespread interest in comparative genomics of birds for both genome evolution studies and comparative mapping in commercial species (BURT *et al.* 1999; SHETTY *et al.* 1999). In recent years several comparative mapping studies have focused on individual chicken macro- and microchromosomes (CROOIJMANS *et al.* 2001; SUCHYTA *et al.* 2001; BUITENHUIS *et al.* 2002; JENNEN *et al.* 2002), expanding and refining the previously described synteny information between chicken, human, and mouse. The use of cross-species chromosome painting is well established as a quick means of generating comparative genomic data between species and thus chromosome-specific probes from at least one avian species would further this work.

Given this information, it is clear that the concerted effort to complete and publish the whole chicken genome sequence is a priority (SCHMID *et al.* 2000; BURT and POURQUIE 2003). This is imminent and will provide an important anchor species between fish and mammals. The ability to distinguish all chicken chromosomes ($2n = 78$) is a crucial step in this project as, without it, many genes cannot have proper assignments. Moreover, such a resource has a range of other applications. In this article therefore we describe the isolation of unique chromosome identifier probes for each chicken chromosome either by mapping of individual clones or by chromosome painting.

MATERIALS AND METHODS

Cell culture and chromosome preparation: Metaphase preparations were generated by standard protocols using chicken

fibroblast cell cultures established from 5- to 7-day-old embryos (GRIFFIN *et al.* 1999; AHLROTH *et al.* 2000). Cells were examined under phase-contrast microscopy for adequate spreading and absence of cytoplasm.

Fluorescence-activated chromosome sorting: Chromosomes were prepared for flow sorting as described previously (CARTER *et al.* 1992), spun briefly ($100 \times g$ for 1 min to remove any debris), and then the supernatant stained with 2 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Sigma, St. Louis) and 40 $\mu\text{g}/\text{ml}$ chromomycin A3 (Sigma). Bivariate flow karyotypes were generated on a FACStar Plus (Becton Dickinson, San Jose, CA) dual laser flow cytometer equipped with two 5-W argon ion lasers. For chromosomes 1–10 and Z, ~400 chromosomes from each peak in the flow karyotype were flow sorted into a 0.5-ml Eppendorf tube containing water. For some of the medium-sized microchromosomes, single chromosomes were flow sorted into tubes.

Microdissection of microchromosomes: Since microchromosomes are virtually indistinguishable, it was essential to microdissect single chromosomes prior to PCR amplification. Briefly, preparations on coverslips were stained with 10% Giemsa dye and placed on the stage of a Leica inverted microscope. Individual chromosomes were isolated from the coverslip using a glass needle driven by an electronically controlled micromanipulator attached to the microscope. The needle was then broken in a tube containing 10 μl sterile distilled water prior to PCR amplification (MASABANDA and GRIFFIN 2003). For the smallest microchromosomes, prehybridization of total genomic chicken DNA to metaphase preparations was performed prior to microdissection.

DOP-PCR generation of chromosome paints: A primary round of degenerate oligonucleotide primed (DOP)-PCR amplification was performed on these chromosomes to amplify the total DNA (CARTER *et al.* 1992; TELENUS *et al.* 1992). From each of these primary DOP-PCR reactions, 1–2 μl was used as a template for a secondary DOP-PCR amplification incorporating biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics). This facilitated amplification and labeling of the relevant chromosome, thus making a chromosome paint (CARTER *et al.* 1992; TELENUS *et al.* 1992).

Labeling of bacterial artificial chromosomes and cosmids: For individual clone mapping experiments, clones were labeled by nick translation (MASABANDA *et al.* 1998) using biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics).

Fluorescent *in situ* hybridization: This was performed following the protocol of MASABANDA and GRIFFIN (2003). Briefly, metaphase preparations were aged for 3 hr at 55°. Labeled probe of 100 ng was dissolved in hybridization buffer (containing 50% formamide, 2 \times SSC, and 10% dextran sulfate). Chromosomes and probe were brought into contact under a 18 \times 18-mm glass coverslip, sealed with rubber cement, and denatured together on a hot plate for 5 min at 68°. The hybridization was carried out for 12–16 hr.

Following the posthybridization washes (once for 2 min in 0.4 \times SSC/0.3% Igepal at 73°, once for 1 min in 2 \times SSC/0.1% Igepal at room temperature), equilibration for higher salt concentration in 4 \times SSC/0.05% Tween 20, and blocking in 4 \times SSC/0.1% Tween 20/2% BSA, biotinylated probes were detected with Cy3-conjugated streptavidin (1:300 dilution in 4 \times SSC, 0.1% Tween 20, 1% BSA), digoxigenin-labeled paints with FITC-conjugated antidigoxigenin (1:50 dilution). Finally, chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield antifade medium before microscope analysis.

For the 11-color fluorescent *in situ* hybridization (FISH) experiment, chromosomes 1, Z, 6, 8, and 10 (pool 1) were labeled directly with Cy3-dUTP (Amersham, Buckingham-

TABLE 1
Summary of chromosome probes identifying each chromosome

Group	Chromosome no.	Chromosome paint isolated?	BAC (or cosmid) isolated?	Linkage group	Marker
A	1	+	+	NA	
	2	+	+	NA	
	3	+	+	NA	
	4	+	+	NA	
	5	+	+	NA	
	6	+	+	NA	
	7	+	+	NA	
	8	+	+	NA	
	9	+	+	E36C06W08	MCW0134
	10	+	+	E29C09W09	ADL0112
B	11	+	+	E30C14W10	LEI0143
	12	+	+	E16C17W22	MCW332
	13	+	+	E48C28W13W27	GCT907
	14	—	+	E35C18W14 + C37	GCT903
	15	—	+	E18C15W15 + C37	GCT14
	16	—	+	NOR chromosome	MCW371
C	17	—	+	E41W17 + C24	ADL0293
	18	+	+	E31E21C25W12	FASN
	19	+	+	E52W19	ACACA
	20	—	+	E47W24 + E32	ADL324
	21	—	+	E54	PLOD
	22	—	+	E38	TVSB3
	23	+	+	E27C36W25W26	MCW249
	24	+	+	E49C20W21	GCT905
	25	+	—	—	^a
	26	+	+	E60E04W23	GCT906
	27	+	+	E59C35W20	GCT22
	28	+	+	E53C34W16	GCT904
	29	+	+	E62	ROS0257
	30	+	+	E65	ROS0263
D	31	+	+	E64	ROS0264
	32	—	+	E25C31	—
	33	+	—	—	—
	34	+	—	—	—
	35	+	—	—	—
	36	+	—	—	—
	37	+	—	—	—
	38	+	—	—	—
A	Z	+	—	NA	
A	W	+	—	NA	

Markers and linkage groups are indicated where known (adapted and expanded upon from FILLON *et al.* 1998). NA, not applicable.

^a Chromosome 25 paint was isolated 11 times following microdissection and flow-sorting experiments. Each time the paint was bright and specific, leading us to conclude that this chromosome consists mostly of highly repetitive sequences.

shire, UK); chromosomes 2, 5, 6, 8, and 9 (pool 2) were labeled directly with Cy5-dUTP (Amersham); chromosomes 3, 5, 7, 8, and 10 (pool 3) were labeled with biotin-16-dUTP (Roche Diagnostics); chromosomes 4, Z, 7, 9, and 10 (pool 4) were labeled with digoxigenin-11-dUTP (Roche Diagnostics). This was achieved first by combining the respective primary PCR products for each pool [6 μ l for chromosomes 1 and 2, 4 μ l for chromosomes 3, 4, 5, 6, and Z, and 2 μ l for the rest (10 μ l = \sim 1 μ g)], ethanol precipitating, and resuspending in 10 μ l of water. Next, each pool was labeled by incorporating the relevant dUTP label in the secondary DOP-PCR as de-

scribed above. Secondary DOP-PCR products were pooled (10 μ l for pool 1, 10 μ l for pool 2, 6 μ l for pool 3, and 5 μ l for pool 4), ethanol precipitated with an excess of chicken cot-1 DNA, and resuspended in hybridization buffer. FISH proceeded as above except that biotin-labeled probes were detected with a Cy3.5-avidin conjugate (Amersham). As before, digoxigenin-labeled probes were detected using FITC-conjugated antidigoxigenin antibody (Roche Diagnostics).

Microscope analysis was performed using a Leica DM epifluorescence microscope and images captured with a Photometrics CCD camera attached to the microscope, using ei-

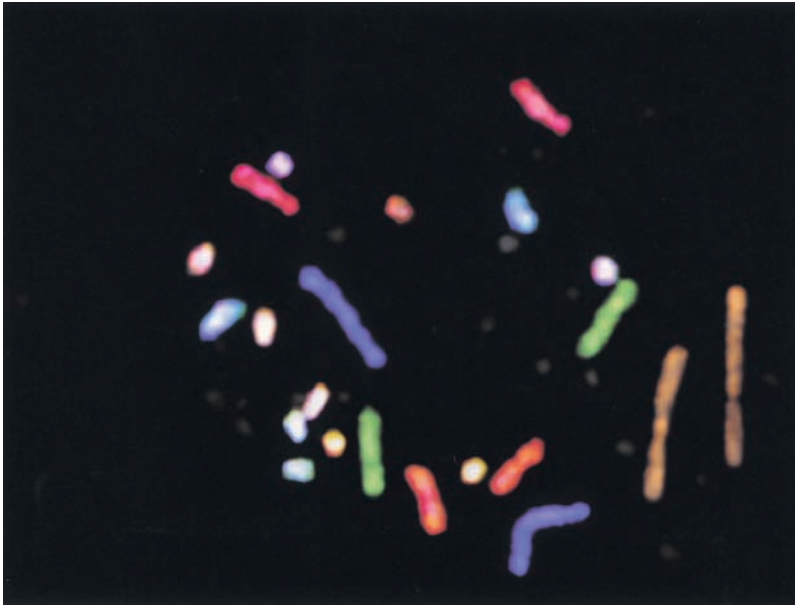


FIGURE 1.—Eleven-color chromosome painting of “A group” chromosomes. Chromosome 1, orange/brown; chromosome 2, blue; chromosome 3, green; chromosome 4, red; Z chromosome, orange/red; chromosome 5, light blue; chromosome 6, white; chromosome 7, pale turquoise; chromosome 8, pale brown; chromosome 9, pale yellow; chromosome 10, pale purple.

ther the Vysis/Digital Scientific “Smart Capture” software or the Leica Q-FISH software for the 11-color experiments.

RESULTS

In this study, we have isolated and assigned at least one chromosome paint or locus specific to each of the 40 chicken chromosomes (Table 1). We made chromosome paints for chromosomes 1–10, Z, and W by flow sorting ~400 chromosomes and DOP-PCR (Figure 1 and MATERIALS AND METHODS). For chromosomes 11–32 (except 25) we identified at least one locus-specific FISH probe associated with a known linkage group and all but chromosomes 14–17, 20–22, and 32 are characterized by a chromosome paint isolated by flow sorting or microdissection of a single microchromosome followed by DOP-PCR. Dual-color hybridization of paints and bacterial artificial chromosomes (BACs; Figure 2) permitted assignment of paints to known linkage groups. For chromosomes 33–38, prehybridization of genomic DNA to metaphases followed by microdissec-

tion and DOP-PCR isolated seven unique paints for the smallest chromosomes that do not cohybridize with one another or with any probe from chromosome 20 to 32 (*e.g.*, Figure 3).

DISCUSSION

To the best of our knowledge, this is the first report of a complete karyotype of any avian species and a critical step for the completion of the chicken genome map. Presently microchromosomes are identified in terms of genetic linkage groups following experiments performed in East Lansing, Michigan, Compton, United Kingdom, and Wageningen, The Netherlands, and thus assigned number(s) are preceded by the letter E, C, and/or W. Here we associated our probes with most known linkage groups (FILLON *et al.* 1998; CROOIJMANS *et al.* 2000), but for chromosomes 25 and 33–38 no linkage was established (Table 1). A priority therefore will be to sequence tag these chromosomes by isolation

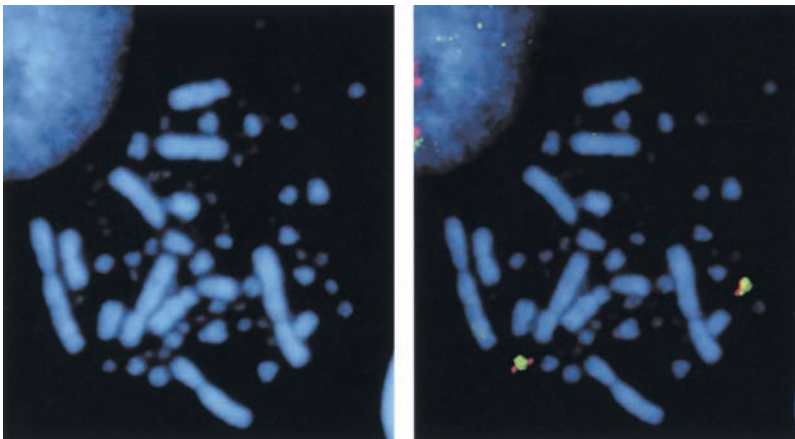


FIGURE 2.—Dual-color hybridization of chromosome paint for chromosome 18 (green) and BAC (red) from linkage group E31E21C25W12 to illustrate how chromosome paints were assigned linkage groups.

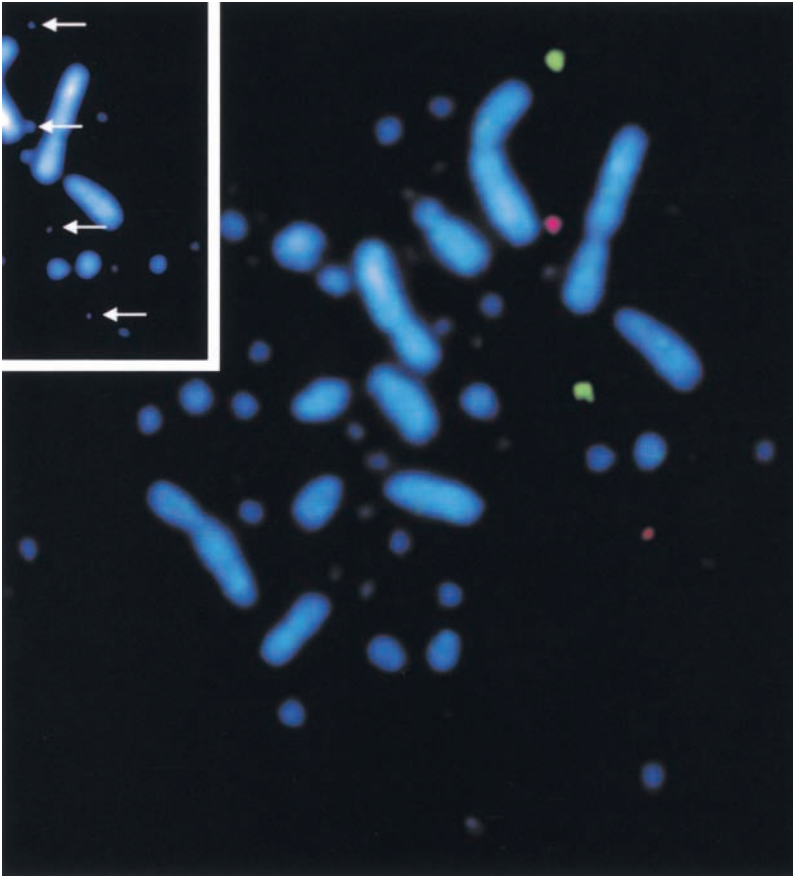


FIGURE 3.—Dual-color chromosome painting of chromosomes 37 (red) and 38 (green). The inset (50% size) indicates DAPI images of chromosomes without the paints covering them.

of genomic DNA from the chromosome paints, sequencing, cloning into BAC or cosmid vectors, and rehybridizing simultaneously with the original chromosome paint by dual-color FISH to confirm the origin of the clone. Preliminary data suggest that clones map back to the original chromosomes in this way in ~20% of experiments. Thus we estimate that to isolate clones and establish linkage groups and/or sequence tags for all remaining microchromosomes, 1–2 more years of experimentation are necessary.

The ability to distinguish each chicken chromosome is also key to the development of genome maps in other avian species. Cross-species FISH is a well-established means of generating low-resolution physical gene maps (WIENBERG and STANYON 1995; O'BRIEN *et al.* 1997). This commonly makes use of human chromosome paints on the metaphases of other mammals but, to date, has been applied relatively rarely to nonmammalian vertebrates (SUZUKI *et al.* 1999; NANDA *et al.* 2000; SUCHYTA *et al.* 2001). Priority avian species for comparative gene mapping are those of commercial interest, *e.g.*, turkey, goose, duck, and quail. Less common species, however, warrant investigation and our macrochromosome paints have already found utility in establishing that, in contrast to mammals, avian chromosomes are remarkably conserved throughout evolution (SHETTY *et al.* 1999;

SUZUKI *et al.* 1999). Similar experiments with microchromosomes are now possible.

Chicken chromosome paints are essential for the characterization of aberrant avian karyotypes, *e.g.*, chromosomally abnormal individuals or aberrant cell lines. They have been used in the characterization of functional domains in the BRCA2 gene (WARREN *et al.* 2002) and for DT40 karyotyping (FUKAGAWA *et al.* 1999). Detecting chromosome aberrations in DT40 can be a key step in addressing questions of genome stability, DNA repair, gene expression, cell death, cell division, and nondisjunction. In the postgenomic era, it is becoming increasingly apparent that three- and four-dimensional genome organization in the interphase nucleus is central to development and disease. Assaying for the position of chromosome territories is a well-established means of establishing genome organization and our chromosome probes have made it possible to begin to study structure and arrangements of chromosome territories in chicken cells (HABERMANN *et al.* 2001). In so doing, evolutionary conserved principles of genome organization have been established.

The classification of chicken chromosomes varies in the literature. Depending on definitions given by different authors, chicken has been reported as having between 6 and 10 pairs of macrochromosomes (SMITH

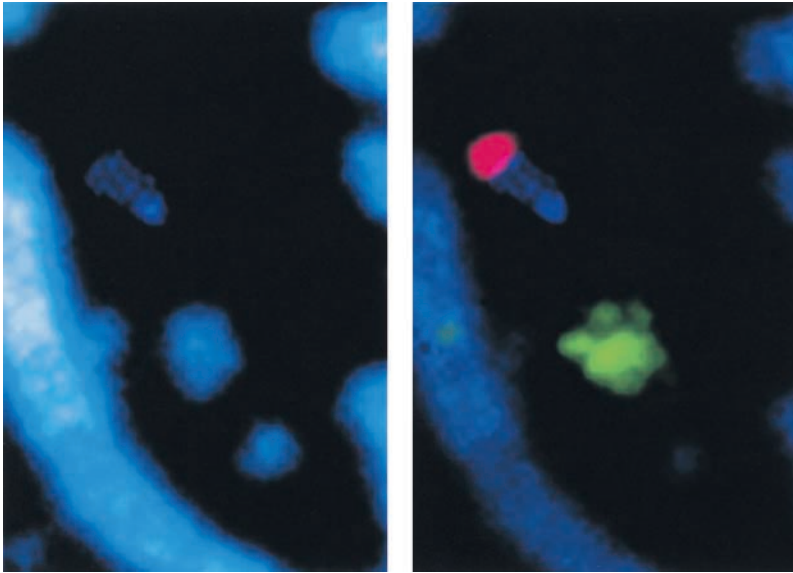


FIGURE 4.—Dual-color experiment using a chromosome paint for chromosome 19 (green) and BAC BW065G09 (for the NOR chromosome 16, red) to illustrate the size differential. Pixel measurements of the relative sizes of the two chromosomes indicate that chromosome 19 is an average of 1.25 times larger than chromosome 16; however, it is clear that the majority of the chicken genome mapping community do not favor a change of assignment.

and BURT 1998; LADJALI-MOHAMMEDI *et al.* 1999; SMITH *et al.* 2000). AUER *et al.* (1987) were able to distinguish chromosomes 1–18 by G-banding and named the NOR chromosome number 17 using silver staining. MILLER *et al.* (1996) and WAIN *et al.* (1998), however, named it chromosome 16, and, in the majority of publications, this dogma is maintained wherever the chromosome is given an assignment at all. Our own pixel measurements of the size of DAPI signals from the NOR chromosome compared to the others in >20 different individuals indicated that it is significantly smaller than chromosome 19 (Figure 4). We propose that previous assignments (AUER *et al.* 1987; MILLER *et al.* 1996; WAIN *et al.* 1998) overestimated the size of the chromosome because the silver staining preferentially recognizes this chromosome, making it appear larger. Nevertheless, we propose that the assignment of 16 should remain to avoid the confusion that would arise from reassigning it at this late stage. From discussions with colleagues, it seems that this is the view of the majority of the chicken genome mapping community. In Table 1, we thus propose the definitive classification system. Group A is composed of chromosomes 1–10, Z, and W (cytogenetically distinguishable macrochromosomes tractable in a flow karyotype). Group B is composed of chromosomes 11–16 (large microchromosomes up to and including the NOR chromosome). Group C is composed of chromosomes 17–32 (small microchromosomes associated with known linkage groups) and group D chromosomes 33–38 (smallest microchromosomes not associated with known linkage groups at the time of writing). This classification builds on our own previous work and that of Vignal, Fillon, and colleagues (FILLON *et al.* 1998; GRIFFIN *et al.* 1999; SCHMID *et al.* 2000). In particular, the assignments of the group B and C chromosomes and their linkage groups follow those of SCHMID *et al.* (2000).

In conclusion, this study is the first to classify the smallest of the chicken chromosomes and to provide a complete karyotype of any avian species. This is a critical step in the completion of the chicken genome map and the resources developed here will have a wide range of applications.

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